

Cellular and Humoral Immune Abnormalities in Gulf War Veterans

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We examined 100 symptomatic Gulf War veterans (patients) and 100 controls for immunologic assays. The veterans and controls were compared for the percentage of T cells (CD3); B cells (CD19); helper:suppressor (CD4:CD8) ratio; natural killer (NK) cell activity; mitogenic response to phytohemagglutinin (PHA) and pokeweed mitogen (PWM); level of immune complexes; myelin basic protein (MBP) and striated and smooth muscle autoantibodies; and antibodies against Epstein-Barr virus, cytomegalovirus, herpes simplex virus type 1 (HSV-1), HSV-2, human herpes Type 6 (HHV-6), and *Varicella zoster* virus (VZV). The percentage of T cells in patients versus controls was not significantly different, whereas a significantly higher proportion of patients had elevated T cells compared with controls. The percentage of B cells was significantly elevated in the patients versus the controls. The NK cell (NK) activity was significantly decreased in the patients (24.8 ± 16.5 lytic units) versus the controls (37.3 ± 26.4 lytic units). The percentage of patients with lower than normal response to PHA and PWM was significantly different from controls. Immune complexes were significantly increased in the patients (53.1 ± 18.6 , mean \pm SD) versus controls (34.6 ± 14.3). Autoantibody titers directed against MBP and striated or smooth muscle were significantly greater in patients versus controls. Finally, the patients had significantly greater titers of antibodies to the viruses compared with the controls ($p < 0.001$). These immune alterations were detected 2–8 years after participation in the Gulf War. The immune alterations are consistent with exposure to different environmental factors. We conclude that Gulf War syndrome is a multifaceted illness with immune function alterations that may be induced by various factors and are probably associated with chronic fatigue syndrome. **Key words:** autoantibodies, B cell, Gulf War syndrome, immune complexes, natural killer cell, T cell. *Environ Health Perspect* 112:840–846 (2004). doi:10.1289/ehp.6881 available via <http://dx.doi.org/> [Online 17 February 2004]

Veterans returning from the Gulf War of 1990–1991 described multiple-organ symptoms that included chemical sensitivity, chronic fatigue, fibromyalgia, neurocognitive deficits, irritable bowel syndrome, headaches, joint pains, fever, and a general unwellness, referred to as Gulf War syndrome (GWS) (Cherry et al. 2001; Gray et al. 2002; Haley and Kurt 1997; Hallman et al. 2003; Iowa Persian Gulf Study Group 1997; Kang et al. 2002; Kroenke et al. 1998; Lashof and Cassells 1998). GWS probably has a multiple-factorial etiology involving possible chemical exposures (Haley and Kurt 1997). The suspected factors include Arabian sand dust, low-level chemical and biological warfare agents, pyridostigmine bromide, *N,N*-diethyl-*m*-toluamide, organophosphate pesticides, depleted uranium, combustion by-products of oil well fires, diesel exhaust, petroleum products, and infectious agents (Abou-Donia et al. 2001; Kurt 1998; Moss 2001; Schumm et al. 2002; Sharabi et al. 1991). Explanations for GWS include genetic polymorphism of para-oxonase (PON1) (Haley et al. 1999; La Du et al. 2001), butyrylcholinesterase (Shen 1998), glutathione *S*-transferase and cytochrome P450_{1A1} (Whatt et al. 2000), and, more recently, neuropathy target esterase (Winrow et al. 2003). Clinical findings include abnormalities of basal ganglia and brainstem (Haley et al. 2000) and recently reported increased neurologic diseases and, particularly, an

increased rate of amyotrophic lateral sclerosis (ALS) (Haley 2003; Horner et al. 2003). Moreover, Gulf War Veterans have an increase in serum RNAs with segments homologous to chromosome 22q11.2 ALU, indicating a rearrangement of this antigen-responsive spot (Urnovitz et al. 1999).

The symptoms of GWS are remarkably similar to those of chronically ill individuals with immunologic alterations after exposure to chlorinated pesticides (McConnachie and Zahalsky 1991, 1992), chlorpyrifos (Thrasher et al. 1993, 2001), formaldehyde (Thrasher et al. 1987, 1990), silicone breast implants (Vojdani et al. 1993), and solvents (Vojdani et al. 1992). Reported immunologic abnormalities suggest a shifting of the immune system to T_H1 and/or T_H2 profiles (Ferguson and Cassaday 2001/2002; Patarca 2001; Rook and Zumia 1997; Zhang et al. 1999). Moreover, levels of complement components (C3a, C4a, C5a) are significantly increased in chronic fatigue syndrome (CFS) patients versus controls (Sorensen et al. 2003). As a result, we were asked to examine symptomatic GWS patients to determine if they had immunologic alterations similar to those seen in CFS (Levine et al. 1998; Patarca 2001; Rosenbaum et al. 2002), chemically exposed individuals (Baj et al. 1994; Cooper et al. 2002; Griem et al. 1998; Thrasher et al. 1993; Vojdani et al. 1992), and earlier reports of GWS (Rook and Zumia 1997).

Materials and Methods

Subjects. Immunosciences Lab., Inc. was approached by R. Haines of the U.S. Army requesting immune testing of 10th Army Unit Gulf War Veterans who had GWS with common symptoms (joint pain, fatigue, headache, memory or concentration difficulties, sleep disturbances, and rash). Haines, with the help of medical doctors, initially selected 50 symptomatic patients (group A), followed at a later date by an additional 50 symptomatic patients (group B). Medical records and health histories were not forwarded for us to review. Controls consisted of two groups: group C consisted of 50 asymptomatic vaccinated veterans of the 10th Army Unit who were not deployed to the Persian Gulf, and group D consisted of 50 healthy asymptomatic subjects sent to the laboratory by treating physicians for annual checkups. Patients ranged from 34 to 56 years of age and consisted of 74 males and 26 females. The controls were age and sex matched. Venous blood was received within 24 hr from different clinics from several states and immediately processed for immunologic testing. Groups A and C underwent immunologic testing in 1993. The testing of groups B and D occurred over an extended period from 1995 through 1999. The tests were done at no charge to the U.S. Army and as a result were limited to immune parameters presented below. The test requests were properly documented and kept in a confidential file. All subjects gave their informed consent and allowed inclusion of their data in this report without disclosure of their identity in the publication.

Basic laboratory tests. Patients and controls underwent complete blood counts (CBC); chemistry, including lipids, liver enzymes, thyroid function [triiodothyronine

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Portions of this study were presented by testimony of A.V. before the Senate Subcommittee on Veterans Affairs 16 November 1993 and the Presidential Special Oversight Board 19 October 1999.

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The authors declare a competing financial interest in that this study was conducted with no charge to the soldiers. Also, A.V. is a co-owner of the laboratory, and J.D.T. serves as a paid consultant.

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(T₃), thyroxine (T₄), thyroid-stimulating hormone (TSH); and basic immunology [antinuclear antibody (ANA), rheumatoid factor (RF), and total IgA, IgG, and IgM].

Lymphocyte subset enumeration. Direct immunofluorescence staining of cell surface antigen was accomplished using the Becton-Dickinson FACScan Immunocytometry system (San Jose, CA). The peripheral mononuclear cells are treated with monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE). The blood samples were first treated with red blood cell lysing solution. Cells were then washed, stained with monoclonal antibody, and then analyzed with the FACScan flow cytometer. We used the following pairs of FITC- or PE-conjugated monoclonal antibodies: CD45/CD14 for quality control check, CD3/CD4 for T-helper cells, CD3/CD8 for suppressor cells, CD3/CD19 for T and B cells, and CD3/CD16 plus CD56 for natural killer (NK) cells (NKHT3⁺ and NKHT3⁻). Using these sets of monoclonal antibodies, we determined the percentage of positively stained cells for each marker pair and the percentage of double stained cells.

Preparation of peripheral blood leukocytes. Leukocytes were prepared from heparinized peripheral venous blood by Ficol-Hypaque density gradient (Sigma Chemical Company, St. Louis, MO). Cells were washed three times with Hanks balanced salt solution and resuspended to a concentration of 10⁷ cells/mL in a complete medium (CM) that consisted of RPMI-1640 supplemented with 10% fetal calf serum and 1% antibiotics (100 U penicillin and 100 µg/mL streptomycin). We examined purity of the cells by flow cytometry using CD45/CD14 monoclonal antibodies; purity was > 95%. Cells were used for different functional assays within 1 hr of isolation.

NK cell cytotoxicity assay. We used a standard 4-hr ⁵¹Cr-release assay (Whiteside et al. 1990) to determine NK cell cytotoxicity. Briefly, we added 1 × 10⁴ ⁵¹Cr-labeled K562 tumor target cells in 0.1 mL CM to different wells of a microtiter plate. Effector cells were then pipetted into triplicate wells for each dilution to give effector:target ratios of 6:1, 12:1, 24:1, and 48:1. After a 4-hr incubation at 37°C, the plates were centrifuged at 1,400 rpm for 4 min, and 0.1 mL of supernatant was collected from each well and placed in a gamma counter. The percentages of isotope released were calculated using the following formula:

$$\text{Percentage of lysis} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Total release} - \text{Spontaneous release}} \times 100 \quad [1]$$

The results of NK cell assay for each effector:target ratio was expressed in terms of

lytic units (LU), calculated as described by Whiteside et al. (1990).

Lymphocyte mitogenic assay. Lymphocytes were isolated and tested for mitogenic activity as described by Fletcher et al. (1992) and Maino et al. (1995). Briefly, 5 × 10⁵ lymphocytes per 0.1 mL CM were cultured in flat-bottom microtiter plate wells. Cells from patients and controls, as well as cells with known mitogenic stimulation, were cultured with or without an optimal concentration of either phytohemagglutinin (PHA; 25 µg/mL; Grand Island Biological Company, Grand Island, NY) or pokeweed mitogen (PWM; 5 µg/mL; Sigma Chemical Company). After 48 hr of incubation, the cells were harvested and stained with CD69 monoclonal antibody conjugated to fluorescent dye and analyzed by flow cytometry. Wells with no mitogens added (negative control) provided information about the media and cells used in the assay so we could determine possible nonspecific modulatory activity. Values for patients and controls were compared with the daily negative and positive control for each assay. Results were calculated using the following formula:

$$\text{Percentage of stimulation} = \frac{\text{Activated sample} - \text{Unstimulated sample}}{\text{Activated control} - \text{Unstimulated control}} \times 100 \quad [2]$$

We estimated three stimulation levels: low, < 75% of the number of control cells; normal, 75–125% of the number of control cells; and elevated, > 125% of the number of control cells.

Myelin basic protein antibody. The antibody to human myelin basic protein (MBP) was analyzed as previously described (Vojdani et al. 1992). Briefly, MBP (Sigma Chemical Co.) was checked for purity by polyacrylamide gel electrophoresis (Diebler et al. 1972). Antisera to MBP were induced in rabbits by repeated injection of the protein in complete Freund's adjuvant. Antibody activity in the rabbit sera and the patient and control samples was detected by adding different dilutions (1:100–1:10,000) of sera to wells of a microtiter plate previously coated with MBP. MBP (250 µg/mL) was dissolved in carbonate buffer (pH 9.6), and 200 µL of this solution was added to each well. After incubation, washing, and blocking with 1.5% bovine serum albumin plus gelatin, 200 µL of either diluted rabbit or human serum was added to the wells. Incubation was repeated for 1 hr at 25°C, and the sera were shaken out of the wells, which were then washed five times with wash solution. Next, 200 µL peroxidase-conjugated goat anti-rabbit or goat anti-human IgM (optimal dilution) was added to the appropriate well. After incubation and repeated washing, 200 µL substrate was added

to each well. Plates were incubated for 1 hr at room temperature and read in a microtiter reader at 480 nm wavelength. We plotted a titration curve using rabbit antisera, and compared patient and control sera with this standard curve. Results are expressed by the ELISA (enzyme-linked immunosorbent assay) values.

Striated and smooth muscle antibody assay. We purchased skeletal muscle myoblast cell line CRL-1769 and smooth muscle cell line ATCC CRL-1444 from American Type Culture Collection (Rockville, MD). Cells were grown in Dulbecco's modified Eagle's medium (90%) and fetal bovine serum (10%). After 7 days in culture, the cells were harvested, sonicated, and used for coating ELISA plates. Each ELISA microplate well was coated with 100 µL cell lysate containing 10 µg protein in 0.1 M carbonate buffer (pH 9.5). Plates were incubated overnight at 4°C and then washed three times with 200 µL Tris-buffered saline containing 0.05% Tween 20 (pH 7.4). With the exception of goat anti-human IgG F(ab')₂, all other steps were similar to the MBP ELISA method described above. To detect nonspecific binding, we used all reagents except human serum in several control wells and coated some wells with different tissue antigens.

Immune complexes. We measured IgG, IgM, and IgA immune complexes by coating microtiter plates with anti-C1q. Addition of serum, second antibody, and substrate was similar to procedures for measuring MBP antibody by ELISA.

Antibodies to viruses. Antibody titers against Epstein-Barr virus (EBV); cytomegalovirus (CMV); herpes simplex virus type 1 (HSV-1), HSV-2, HHV-6; and *Varicella zoster* virus (VZV) were measured by ELISA using kits from Diamedix (Miami, FL).

Determination of expected ranges. We obtained expected ranges as follows:

- Earlier publications.
- Suppliers of the CD markers (Becton-Dickinson) carried out in-house verification using blood from 100 healthy controls from which the means, SDs, and 95% confidence intervals (CIs) were determined (Babcock et al. 1987, Shearer et al. 2003).
- The manufacturers of the immune complex and viral antibody test kits provided an expected range with their products (Abbas et al. 1994, Christenson et al. 1992, Matheson et al. 1990, Shehab and Brunell 1983).
- We obtained expected ranges for NK cell activity from Whiteside et al. (1990) and confirmed them in our laboratory by performing the assay on 500 specimens from healthy subjects.
- We determined expected ranges for PHA and PWM mitogenesis and for autoantibodies to MBP and striated and smooth muscle in-house using 100 healthy controls for which means, SDs, and 95% CIs were calculated.

Thus, the expected ranges are a combination of suppliers' recommendations and in-house quality control.

Statistics. We used two-sided critical *t*-tests for comparison of independent means and *Z*-tests for comparison of independent proportions as described by Bourke et al. (1985).

Results

Basic laboratory tests. We observed some variations from expected normal ranges in both controls and patients, respectively, as follows: CBC, 21 and 27%; blood glucose, 12 and 9%; lipid profile, 18 and 22%; liver enzymes, 14 and 19%; T₃, T₄, and TSH, 9 and 12%; ANA, 7 and 11%; RF, 10 and 14%; and immunoglobulins, 6 and 8%. *Z*-tests for independent proportions revealed no significant difference between controls and patients for each test.

Comparison of patients (groups A and B) and controls (groups C and D). We analyzed the data to determine if differences existed between patient groups A and B and their controls for each tested immune parameter (data not shown). No significant differences were found; therefore, we combined patient groups A and B and control groups C and D for further statistical analysis. In addition, we observed no differences between males and females in any of the groups.

CD3 T cells. The percentages of CD3 T cells present in the peripheral blood of controls versus patients are presented in Table 1. The mean percentage of CD3 cells in patients ($72.8 \pm 10.3\%$, mean \pm SD) was slightly higher compared with the controls ($71.6 \pm 7.3\%$), but the difference was not significant. However, the percentage of individuals that fell outside of the expected range of 53–79% CD3 cells was different between the two groups; 2% of controls and 9% of patients had < 53% CD3 cells, and 5% of controls and 30% of patients had > 79%. The critical *Z*- and *p*-values for variance from normal distribution were significant ($p < 0.05$).

CD19 B cells. The percentages of CD19 B cells present in the peripheral blood of controls versus the patients are presented in Table 1. The mean percentage of B cells in the patients ($16.1 \pm 6.0\%$, mean \pm SD) was greater than that for the controls ($11.5 \pm 3.1\%$). The difference between the two means was highly significant ($t = 11.402$, $p < 0.001$). The distribution of B cells outside of the expected range (5–15%) was also different when the patients were compared with the controls. Forty-nine percent of patients and 5% of controls had > 15% B cells, whereas 0% of controls and 4% of patients had < 5% B cells. The critical *Z*-values for variance from normal distribution were significant ($p < 0.01$).

CD4:CD8 ratio. The CD4:CD8 (helper:suppressor) ratios for controls and

patients are listed in Table 1. The ratio was significantly ($p < 0.001$) elevated in patients (2.23 ± 0.87 , mean \pm SD) compared with controls (1.74 ± 0.34). The *Z*-values for the percentage of patients with values outside the expected distribution of the CD4:CD8 ratio were significantly different compared with those of the controls ($p < 0.001$).

NK cell activity. The data obtained for NK lytic activity are presented in Table 2. The lytic activity was significantly less ($p < 0.001$) in patients (24.8 ± 16.5) than in controls (37.3 ± 26.4). The percentages of individuals with > 50 LU were not different between the two groups ($p =$ not significant), whereas the *p*-value for the percentage of patients with < 20 LU was significant ($p < 0.01$).

Mitogen stimulation. The results of PHA and PWM stimulation of peripheral lymphocytes in the controls and patients are summarized in Table 2. The mean percentage of stimulation by either PHA or PWM was not different between controls and patients. However, the distribution of stimulation values did differ between the two groups. For PHA, more GWS patients (32%) had a stimulation

index < 75% of expected ($p < 0.01$) but 4% of patients had a stimulation index > 125% ($p < 0.05$). For PWM stimulation, an increased percentage of patients had an index < 75% ($p < 0.01$), whereas the difference between the percentages of patients (4%) and controls (1%) with values > 125% were not significant.

Autoantibodies. The observations for IgM anti-MBP in the controls and patients are shown in Table 3. The mean ELISA units for IgM antibodies were significantly ($p < 0.001$) greater in patients (45.9 ± 35.8) than in controls (28.4 ± 13). The *Z*-value for the percentage of patients with > 50 ELISA units was significant ($p < 0.001$). The mean value for the patients with IgM titers > 50 ELISA units was 75.7 ± 15.9 .

Antibodies to muscle (striated and smooth). The results for IgG antibodies to both smooth and striated muscle are shown in Table 3. The mean IgG titers observed in the patients (42.8 ± 72.3) were significantly ($p < 0.001$) greater than those for the controls (15.9 ± 11.4).

Immune complexes. The observations on immune complexes found in the peripheral blood of controls and patients are summarized

Table 1. Percentage of CD3 T cells and CD19 B cells and CD4:CD8 ratios in controls and patients.

Cell type or ratio	Percent (mean \pm SD)	Expected range (%)	Percent of subjects outside the expected range	
			Below bottom of range	Above top of range
CD3 T cells				
Controls	71.6 \pm 7.3	53–79	2	5
Patients	72.8 \pm 10.3	53–79	9	30
			<i>Z</i> = 2.17 <i>p</i> < 0.05	<i>Z</i> = 4.65 <i>p</i> < 0.01
CD19 B cells				
Controls	11.5 \pm 3.1	5–15	0	5
Patients	16.1 \pm 6.0	5–15	4	49
			<i>Z</i> = 6.38 <i>p</i> < 0.01	<i>Z</i> = 11.4 <i>p</i> < 0.001
CD4:CD8 ratio				
Controls	1.74 \pm 0.34	1.0–2.5	0	3
Patients	2.23 \pm 0.87	1.0–2.5	4	33
			<i>Z</i> = 2.2 <i>p</i> < 0.05	<i>Z</i> = 5.33 <i>p</i> < 0.001

Controls and patients were compared using Student's *t*-test. Critical *Z*-values were obtained for the normal distribution.

Table 2. NK cell activity and lymphocyte stimulation with PHA and PWM in controls and patients.

Cell type	Lytic units (LU)		Percent of subjects outside the expected range	
	Percent (mean \pm SD)	Expected range (%)	Below bottom of range	Above top of range
NK cell activity				
Controls	37.3 \pm 26.4	20–50	8	9
Patients	24.8 \pm 16.5	20–50	47	6
			<i>Z</i> = 6.35 <i>p</i> < 0.01	<i>Z</i> = 0.80 NS
PHA				
Controls	84.9 \pm 20.2	75–125	6	0
Patients	84.4 \pm 19.9	75–125	32	4
			<i>Z</i> = 4.6 <i>p</i> < 0.01	<i>Z</i> = 2.02 <i>p</i> < 0.05
PWM				
Controls	85.4 \pm 11.5	75–125	7	1
Patients	87.3 \pm 18.6	75–125	23	4
			<i>Z</i> = 3.16 <i>p</i> < 0.01	<i>Z</i> = 1.35 <i>p</i> < 0.05

NS, not significant. Controls and patients were compared using Student's *t*-test and *Z*-test.

in Figure 1. Immune complexes were significantly ($p < 0.001$) elevated in the patients (52.1 ± 18.5 mEq/mL) compared with controls (34.5 ± 14.3 mEq/mL) (Figure 1A). In addition, the percentage of patients with immune complexes > 50 mEq/mL was significantly higher than that for controls ($p < 0.01$) (Figure 1B).

Antibodies to viruses. The results obtained on antibodies to viruses are presented in Table 4. Mean titer levels for each virus were significantly ($p < 0.001$) higher in the patients than in controls.

The differences in the mean titer levels between the patients and controls resulted from a disproportionate number (percentage) of patients who had elevated titers to each virus. For example, the mean IgM antibodies to EBV viral capsid antigen (VCA) was 724 ± 401 ELISA units for the 56 individuals that fell above the maximum expected titer of 300 ELISA units. Similar observations were evident for each of the remaining viruses. Z-values for the percentage of patients with elevated titers were significantly different from those for controls (Figure 2).

Discussion

The patients and the controls that underwent immunologic testing were from two different time periods, 1993 versus 1995–1999. In addition, the two sets of controls were different: one consisted of asymptomatic U.S. Army soldiers who did not serve in the Gulf War, and the other consisted of healthy individuals undergoing annual checkups. Therefore, we deemed it necessary to determine if there were any differences between the groups. *t*-Tests comparing the means of each immune parameter revealed that no differences existed between the two sets of patients as well as the two sets of controls (data not shown), permitting the pooling into patients ($n = 100$) versus controls ($n = 100$). This analysis revealed two interesting observations: *a*) GWS patients had immunologic alterations approximately 2–8 years after participation in the Gulf War; and *b*) the asymptomatic soldiers' immunologic

findings were no different from those of healthy individuals undergoing an annual checkup.

When we analyzed the data on basic laboratory testing (CBC, chemistry, T_3 , T_4 , TSH, RF, and other measures, the differences between patients and controls were not significant. Therefore, we conclude that such tests are of little value in diagnosing GWS.

One of the fundamental issues facing the field of immunotoxicology is the degree of perturbation to a given parameter after exposure to a xenobiotic that translates into a significant health risk. A number of methods have evolved to address the question of how a chemical or drug affects the ability of the immune system to resist challenge by pathogens or abnormal host cells (e.g., tumor). These include both functional (i.e., activity of one or more specific cell types) as well as host resistance assays. They were designed to evaluate the overall changes in the functional integrity of the immune system associated with certain chemicals, pharmaceuticals, and biotherapeutic molecules (Cornacoff et al. 1995; Loveless et al. 1997; Luster et al. 1988, 1993; Smialowicz et al. 1995). Therefore, analysis of circulating lymphocyte subpopulations reveals that alterations do exist in increased T-cell numbers, B-cell numbers, and CD4:CD8 ratio in these patients (Table 1). An increased CD4:CD8 ratio indicates an increase in inducer helper CD4 relative to CD8 suppressor/cytotoxic cells. These observations are compatible with previous reports on GWS (Zhang et al. 1999) as well as those regarding lymphocyte subset alterations after exposure to xenobiotics (Baj et al. 1994; McConnachie and Zahalsky 1991, 1992; Thrasher and Broughton 2001; Vojdani et al. 1992, 1993).

Further assessment of the immune system of GWS patients was done by measurement of T- and B-cell function and NK cell activity. Although the mean values for mitogenic response to PHA and PWM were not different in patients versus controls, the percentage of individuals with abnormally low mitogenesis was significantly different between the two

groups (Table 2). Such an analysis is appropriate because immune dysregulation can result in either increased or decreased mitogenic response in some individuals. Mitogen stimulation is used clinically to assess cellular immunity in patients with immunodeficiency, cancer, and autoimmunity, as well as to assess the immunotoxic potential of drugs and chemicals in humans and experimental animals (Smialowicz 1995). Similarly, assessment of NK lytic activity also gives valuable information. The lytic activity of NK cells (Table 2) was significantly lower in the GWS patients (24.8 ± 16.5) versus the controls (37.3 ± 26.4). This was also reflected by an elevated number of GWS patients (47%) with low lytic activity of NK cells. Decreased numbers of NK cells have been reported in GWS individuals with chronic fatigue (Zhang et al. 1999). Furthermore, decreased mitogenesis and decreased NK activity have been reported after exposure to xenobiotics and in chronic fatigue (Heuser and Vojdani 1997; Levine et al. 1998; Racciatti et al. 2001; Vojdani et al. 1992, 1993; Whiteside and Friberg 1998). Thus, the observed changes in the functional status of T-, B-, and NK cells in GWS patients are consistent with immune alterations in humans after exposure to xenobiotics as described above.

The most critical but open question in immunosurveillance is whether information on differences between individuals with abnormal NK, T-, and B-cell function can predict future development of cancer or autoimmune diseases. Indications of the significant role for NK cells in preventing the development of cancer in both mice and humans have been reported (Imai et al. 2000; Wilson et al. 2001). A prospective cohort study among a Japanese general population showed that medium and high cytotoxic activities were associated with reduced cancer risk, whereas low activity was associated with an increased risk (Imai et al. 2000). Wilson et al. (2001)

Table 3. IgM antibody against MBP and IgG antibodies against smooth and striated muscle in controls and patients.

Antibody type	Level of IgM antibody		Percent of subjects above top of range
	Mean \pm SD	Expected range (ELISA units)	
Anti-MBP			
Controls	28.4 ± 13	0–50	4
Patients	45.9 ± 35.8	0–50	34
			$Z = 5.57$
			$p < 0.001$
Against smooth and striated muscle			
Controls	15.9 ± 11.4	0–20	9
Patients	42.8 ± 72.3	0–20	37
			$Z = 4.70$
			$p < 0.001$

Controls and patients were compared using Student's *t*-test and Z-test.

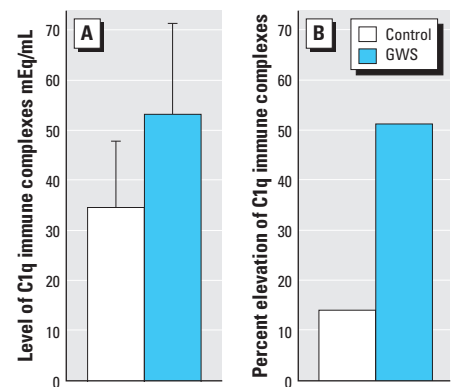


Figure 1. Total level of immune complexes (A) and percentage of elevation (B) in controls and patients with GWS. Error bars indicate SD. Controls and patients were compared using Student's *t*-test. Critical Z-values were also obtained for normal distribution; $p < 0.01$, $Z = 5.58$, *t*-test = 7.46.

demonstrated suppressed NK cell activity with altered host resistance in mice. NK cell activity was incrementally decreased by intravenous administration of antibody to NK surface receptors. After a $\geq 80\%$ decrease in spontaneous NK activity, a challenge with $\geq 1 \times 10^3$ B16F10 melanoma cells resulted in increased tumor burden in the lungs (Wilson et al. 2001). Furthermore, when challenged with 1×10^5 melanoma cells, tumor burden was not increased until spontaneous NK activity had been decreased by $\leq 50\text{--}60\%$. Altered host resistance is a function of both the magnitude of the decrease in NK activity and the magnitude of the challenge to the host (Wilson et al. 2001).

Increased levels of autoantibodies to various organs in humans, including MBPs, have been reported after exposure to toxic chemicals (McConnachie and Zahalsky 1991, 1992; Thrasher et al. 1987, 1990, 1993; Vojdani et al. 1992, 1993). Thus, the increased incidence of antibodies to MBP and striated and smooth muscle (Table 3) in the GWS patients is suggestive of autoimmunity, possibly resulting in tissue injury from toxic chemical exposure (Cooper et al. 2002; Griem et al. 1998). The presence of IgM antibodies to MBP appears to indicate that an active process involving release of these self-antigens is occurring up to 8 years after injury. Central nervous system injury has been reported in research animals exposed to pyridostigmine bromide, DEET (*N,N*-diethyl-*m*-toluamide), and permethrin (Abou-Donia et al. 2001) and in some GWS patients, in particular, ALS (Haley 2003; Horner et al. 2003). The observations presented here suggest that additional studies are needed on neural damage and/or axonal demyelination in symptomatic Gulf War veterans. Neural antigen and MBP antibodies have been reported in patients with neurologic disorders (Terryberry et al. 1998; Willison and Yuki 2002), including ALS (Rogers et al. 1996), autism (Vojdani et al. 2002), and multiple sclerosis (Vojdani et al. 2003). Therefore, our results are consistent with the excess of ALS among Gulf War veterans reported by Haley (2003).

The GWS patients in the present study were found to have significantly elevated circulating immune complexes (Figure 1). Increased immune complexes have not been previously reported to our knowledge in GWS patients. Circulating immune complexes are formed by excessive antigen antibody reaction. Immune complexes have been implicated in numerous immunopathologic conditions, including systemic lupus erythematosus, rheumatoid arthritis, glomerulonephritis, and infectious induced inflammation (Abbas et al. 1994). Deposition of immune complexes can occur from cell- or tissue-specific antibody-antigen reactions, resulting in organ injury

and/or immune complex diseases (Bigazzi et al. 1986). Thus, it would appear from these observations on increased immune complexes in the patient population in the present study that inflammation and autoimmune reactions may exist in a subgroup of patients with GWS. Circulating immune complexes containing IgG, IgM, and IgA antibodies can

generate a variety of substances associated with muscle damage and the acute phase response, activating the classic pathway of complements (Sorensen et al. 2003).

Finally, we tested the GWS patients to determine if an increase in antibodies to several HSV types was present (Table 4, Figure 2). The data clearly show that significantly increased

Table 4. Titer levels of antibodies to viruses found in controls and patients (expressed in ELISA units).

Virus	Controls (mean \pm SD)	Patients (mean \pm SD)	Expected range	t-Test range	p-Value
EBV VCA IgM	197 \pm 166	384 \pm 400	0–300	6.627	< 0.001
CMV IgG	143 \pm 121	271 \pm 277	0–200	4.234	< 0.001
HSV-1 IgG	197 \pm 173	491 \pm 469	0–400	5.881	< 0.001
HSV-2 IgG	144 \pm 162	343 \pm 305	0–400	5.473	< 0.001
HHV-6 IgG	14.9 \pm 8.7	42.6 \pm 54.6	0–20	5.010	< 0.001
VZV IgG	15.4 \pm 11.4	90.5 \pm 149	0–20	4.391	< 0.001

VCA, viral capsid antigen. Controls and patients were compared using Student's *t*-test.

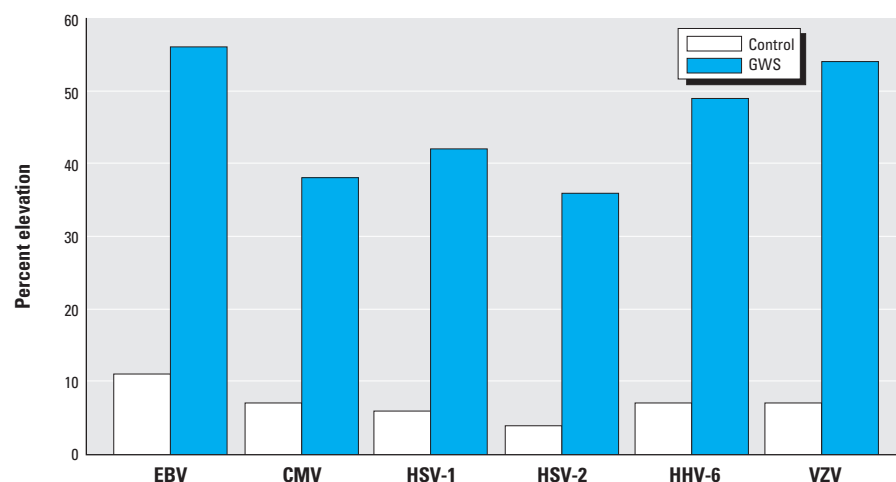


Figure 2. Percent elevation above the expected range of viral antibodies in controls and patients with GWS. $p < 0.01$.

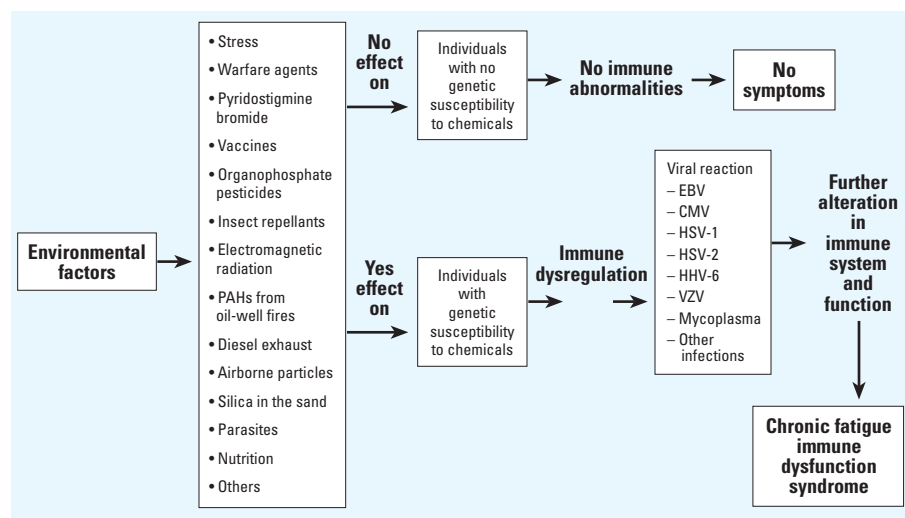


Figure 3. Hypothetical description of GWS in relation to environmental factors present in the Gulf War and their effects on individuals with no genetic susceptibility to chemicals versus those with genetic polymorphism and susceptibility to chemicals resulting in immune function abnormalities and possibly immune dysregulation. Only in the subpopulation susceptible to these environmental factors may these immune abnormalities result in viral reactivation and symptoms similar to those of chronic fatigue and fibromyalgia. PAH, polycyclic aromatic hydrocarbons.

antibody titers occurred in the GWS patients compared with the controls for each virus tested (EBV, CMV, HSV-1, HSV-2, HHV-6, and VZV; Table 4). When the observations were limited to only affected individuals, the increased titers to each virus was even more evident (Figure 2). Exactly when the viral infections occurred cannot be determined from these data. However, the increased IgM antibodies to EBV VCA suggest that reactivation of EBV is probably occurring and may involve the other viruses. To our knowledge, there has been no other report regarding increased viral antibodies in GWS patients. In addition, infections with *Mycoplasma fermentans*, *Mycoplasma hominis*, and *Mycoplasma penetrans* must also be considered (Vojdani and Franco 1999). Although we did not perform polymerase chain reaction to detect DNA of these viruses, the presence of viral antibodies and mycoplasma DNA may be related to dysregulation of the immune system found in this study.

Conclusion

The observations in these GWS patients suggest that an alteration in the number and function of T and B cells and NK cell activity has occurred that may be associated with service in the Persian Gulf. Furthermore, the presence of antibodies to MBP and striated and smooth muscle, increased immune complexes, and increased antibody titers to several DNA viruses indicate that viral reactivation and/or an active inflammatory immune process may be ongoing in some GWS patients (Ferguson and Cassaday 2001/2001; Patarca 2001; Rook and Zumia 1997).

Based on these observations and earlier reports by others, we believe that GWS is a multifactorial disease caused by exposure to a variety of environmental conditions, for example, xenobiotics, vaccinations, and other stressor-related conditions of the Gulf War environment as summarized in Figure 3. We believe that the outlined multiple factors along with genetic susceptibility due to polymorphism of PON1, loss of neuropathy target esterase, glutathione S-transferase, cytochrome P450 enzymes, or other factors may affect some individuals, resulting in immune dysregulation (Haley et al. 1999; Loewenstein-Lichtenstein et al. 1995; Shields 1994; Whatt et al. 2000). These immune functional alterations reported herein may cause viral reactivation and induction of proinflammatory cytokines, resulting in symptoms similar to those of chronic fatigue and fibromyalgia, as well as other symptoms of GWS (Ferguson and Cassaday, 2001/2002; Patarca 2001; Rook and Zumia 1997; Zhang et al. 1999). The variation in individual susceptibility to environmental stresses and toxicants is a new discipline (toxicogenomics), initiated at the National Institute of Environmental Health Sciences, that studies the relationship

between genes and environmental stressors (Waters et al. 2003). This new knowledge of toxicogenomics may enable us to answer why, upon exposure to these environmental factors, some soldiers developed GWS and others did not. Finally, it appears that additional studies involving asymptomatic deployed Gulf War veterans versus symptomatic Gulf War veterans would be beneficial in further understanding the immunologic observations presented herein.

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